Flaviramulus basaltis gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae* isolated from seafloor basalt

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Four yellow-pigmented, Gram-negative, motile strains were isolated from the glassy rind of submarine basaltic lava from the Jan Mayen area of the Norwegian/Greenland Sea at a depth of 1300 m below sea level. The four strains had identical 16S rRNA gene sequences and were indistinguishable in all phenotypic and chemotypic tests performed, indicating that they belonged to the same species. The strains had an obligately aerobic chemo-organotrophic metabolism. The strains were capable of growth at temperatures between -2 and 34 °C, at pH between 6.5 and 8.6, and at sea salt concentrations between 3 and 60 g l⁻¹. The strains were able to utilize organic acids, amino acids and sugars but not alcohols; they were also capable of hydrolysing a wide range of macromolecules. The predominant fatty acids were 15:0 iso, 15:1 iso, 15:0 iso 3-OH and 17:0 iso 3-OH. The mean DNA G + C content of the strains was 31.4 mol%. 16S rRNA gene sequence analysis indicated that the strains were affiliated to the genera *Gaetbulibacter* and *Algibacter*. However, phenotypic characteristics, especially aerobic metabolism, suggested that the strains should be placed within a new genus. On the basis of the polyphasic characterization of the four strains, it is suggested that the strains be included in the family *Flavobacteriaceae* as representatives of a novel species in a new genus, for which the name *Flaviramulus basaltis* gen. nov., sp. nov. is proposed. The type strain is H35^T (= CIP 109091^T = DSM 18180^T).

According to Garrity *et al.* (2005), the family *Flavobacteriaceae* (Reichenbach, 1989; Bernardet *et al.*, 1996, 2002) is placed within the class *Flavobacteria*, one of three classes in the phylum *Bacteroidetes*. Novel members of the family *Flavobacteriaceae* have been described frequently in recent years. Of the 53 genera within the family at the time of writing, 27 were proposed in 2004 or later. Some genera contain species found in soil, freshwater and in association with humans and animals as commensals or as opportunistic pathogens. However, 37 of the genera within the *Flavobacteriaceae*, nearly all proposed since 1997, include species isolated from marine systems. Members of the marine clade (Bowman, 2005) of the family *Flavobacteriaceae* are thought to play an important role in the degradation of complex biological macromolecules in the marine environment (Kirchman, 2002).

Samples of basaltic pillow lavas were collected from the sea floor during the SUBMAR 2001 cruise to the Jan Mayen platform in the Norwegian/Greenland Sea (71° 04' 38" N 7° 29' 6" W). The samples were collected by dredging at a depth of 1300 m. The sea floor temperature at the sampling site

was -0.7 °C. Immediately after the samples were on deck, the outer glassy rim of the pillow basalt was chipped off. The glass was added to sterile seawater and stored in closed 100 ml flasks in darkness for 3 years at 10 °C. Subsamples were plated onto marine broth 2216 (Difco; MB) with 1.5 % agar, and incubated at 10 °C. After 14 days incubation, four yellow colonies were picked. Strains from these four colonies (designated H32, H33, H34 and H35^T) were purified, and preserved in MB supplemented with 15 % glycerol at -80 °C.

Unless stated otherwise the strains were grown in *Flavobacteriaceae* medium (FM; 30 g Sigma sea salts l⁻¹, 5 g D-glucose l⁻¹, 0.5 g yeast extract l⁻¹, adjusted to pH 8.0) at a temperature of 22 °C. Gram staining, oxidase, catalase and alkaline phosphatase tests were performed as described by Smibert & Krieg (1994). The ability of the strains to hydrolyse agar, gelatin, chitin, cellulose (CF11 powder; Whatman), starch, urea, aesculin and DNA was examined according to the procedures of Smibert & Krieg (1994). Hydrolysis of L-tyrosine was examined as described by Collins (1995). Hydrolysis of Tweens was examined as described by Slifkin (2000). The hydrolysis tests were performed on MB + 1.5 % agar (MA) medium at an incubation temperature of 15 °C. FM supplemented with

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Flaviramulus basaltis* H35^T is DQ361033.

cysteine.HCl to a final concentration of 0.05 % was used to examine the ability of the strains to produce H_2S from cysteine (Smibert & Krieg, 1994). FM without glucose and with 0.15 % phenol red was used to test for the production of acids from a variety of sugars supplemented to a final concentration of 1 %. FM with appropriate modifications was used to examine the requirement for growth factors.

Temperature, pH and salinity range and optimum were examined by measuring growth in 96-well microtitre plates. Growth was measured as OD_{630} . To determine the temperature range for growth, the strains were grown in MB. For pH determination, the strains were grown in FM supplemented with 50 mM buffer. The following buffer systems (pH range) were used: citric acid (3.5–4.0), acetic acid (4.5–5.5), PIPES (6.0–7.4), HEPES (7.6–8.0) and Tris (8.2–10.5). For the salinity experiments, FM with different sea salt concentrations was used. The strains were tested for fermentative growth on MB and FM. To test for anaerobic respiration, MB and FM supplemented with $NaNO_3$, Fe(III) citrate or amorphous Mn(IV) oxide to 0.2 % were used. The anaerobic medium was flushed with N_2 immediately after autoclaving to remove O_2 . The medium was then supplemented with $NaHCO_3$ to a final concentration of 0.5 % (w/v) to help facilitate anaerobic growth (Reichenbach, 1989).

Substrate use was examined using Biolog GN2 plates as described by the manufacturer except that the plates were inoculated with cells grown on MA, and suspended in 1.5 % Sigma sea salts to an OD_{590} of ~ 0.3 . The plates were incubated at 15 °C and manually read after 14 days incubation. The ability of the strains to use methanol, ethanol, 2-propanol or glycerol as sole carbon and energy sources was tested using FM as described, but without D-glucose, as the base medium. The medium was then supplemented with the appropriate alcohol to a final concentration of 1 %.

DNA was isolated using the method described by Marmur (1963). DNA G + C content was determined by the thermal denaturation method (Mandel *et al.*, 1970). Fatty acid methyl ester and quinone analyses were carried out by the Identification Service of the DSMZ (Braunschweig, Germany).

16S rRNA genes from the four strains were PCR amplified, sequenced and assembled to a 1422-bp-long fragment by using standard methods. Percentage sequence similarity between the basalt strains and the type species of genera in the family *Flavobacteriaceae* was determined by the global alignment program MATGAT (Campanella *et al.*, 2003). Closely related sequences were selected for generation of phylogenetic trees using the neighbour-joining, maximum-likelihood and maximum-parsimony algorithms. The neighbour-joining tree was constructed in CLUSTAL_X (Thompson *et al.*, 1997) and bootstrap values were calculated based on 1000 replications. Maximum-likelihood and maximum-parsimony trees were constructed in PHYLIP (Felsenstein, 2004).

Pigments were extracted from cells grown without light in MB using ethanol. The cellular residues were removed by centrifugation. An adsorption spectrum (175–900 nm) was obtained from the crude cell extract using a Cary 4E UV–visible spectrophotometer (Varian). Cell pigments were also extracted using acetone, and separated by TLC using cellulose-covered plastic plates (Merck) and petroleum ether/acetone (9:1) as the chromatographic liquid. The separated pigments were cut out and extracted in acetone. An adsorption spectrum (390–700 nm) was obtained from the separated pigments using a UV mini 1240 spectrophotometer (Shimadzu). *Cellulophaga lytica* DSM 7489^T was used as a reference in the pigment analysis (Lewin & Lounsbury, 1969). To detect flexirubin-type pigments, the bathochromic shift test with 20 % (w/v) KOH (Reichenbach, 1989) was performed.

Phenotypic and genotypic analysis could not differentiate the four strains H32, H33, H34 and H35^T. The 16S rRNA gene sequences from the four strains were also identical.

Cells in exponential growth phase were rod-shaped, with a diameter of 0.2–0.3 μm and a length of 1–3 μm . Cells in stationary phase were pleomorphic with a diameter of 0.2–0.3 μm and lengths ranging from 1 to 30 μm . In stationary-phase cultures, branched and curled cells were also observed (Fig. 1). As cultures aged, cells degenerated into spheroplasts ranging from 0.15 to 0.5 μm in diameter (Fig. 1). After 1 month incubation under optimum conditions, nearly all cells had degenerated into spheroplasts, which were found to be non-viable. The strains must, however, be able to survive for at least 3 years at 10 °C on seawater and basalt given that they could be cultivated from the inoculum. Cells displayed gliding motility. Motility was best observed in fresh cultures grown in FM with <0.05 %

Fig. 1. Scanning electron micrograph of cells of strain H35^T from a 2-week-old culture, grown in MB and incubated at 4 °C. Arrows indicate spheroplasts. Bar, 2 μm .

yeast extract. No spreading growth was observed on MA or FM plates. Cells were observed by microscopy to be motile by polar adhesion to the glass slide, with a rotational movement of about 3 rotations s^{-1} . Cells gripped the surface at the opposite pole and then released at the first pole. This behaviour has been described previously for members of the family *Flavobacteriaceae* (Lapidus & Berg, 1982). Polar appendage structures (Fig. 2) were observed by scanning electron microscopy on many of the cells that were motile. These structures were not observed on non-motile cells.

The basaltic strains were obligately aerobic and heterotrophic. They had a temperature range from -2 to $34^{\circ}C$ with an optimum growth rate at $18-23^{\circ}C$. The strains had a pH range from 6.5 to 8.6, with an optimum growth rate at pH 6.5–8.2. The strains grew at $3-60$ g sea salts l^{-1} , with an optimum growth rate at $24-60$ g l^{-1} . The strains required seawater or artificial seawater for growth. The strains also had requirements for thiamine and unknown amino acid(s), but grew well in media supplemented with yeast extract to a final concentration of 0.05 % (w/v). The strains were able to utilize and produce acids from (+)-L-rhamnose, (–)-D-fructose, maltose, sucrose, (+)-D-glucose, (–)-D-arabinose, (+)-D-cellobiose, (–)-D-ribose, xylan, lactose and (–)-D-mannitol. None of the tested alcohols supported growth. The strains were able to use the following substrates in the Biolog system: cyclodextrin, D-glucose, D-glucose 1-phosphate, D-lactose, ketobutyric acid, ketoglutaric acid, methyl D-glucoside, DL-lactic acid, D-cellobiose, dextrin, D-fructose, D-galactose, D-galacturonic acid, D-glucose 6-phosphate, D-glucuronic acid, D-mannose, D-raffinose, D-trehalose, gentiobiose, glycogen, glycyl L-aspartic acid,

glycyl L-glutamic acid, lactulose, L-aspartic acid, L-glutamic acid, L-ornithine, L-proline, L-rhamnose, maltose, *N*-acetyl-D-glucosamine, pyruvic acid methyl ester, succinamic acid, succinic acid, succinic acid monomethyl ester, sucrose, turanose, and Tweens 40 and 80. The following Biolog substrates were not utilized: α -, β - and γ -hydroxybutyric acid, ketovaleric acid, acetic acid, adonitol, bromosuccinic acid, *cis*-aconitic acid, citric acid, D-alanine, D-arabitol, D-galactonic acid lactone, D-gluconic acid, D-glucosaminic acid, D-mannitol, D-melibiose, D-psicose, D-saccharic acid, D-sorbitol, formic acid, glucuronamide, hydroxy-L-proline, i-erythritol, itaconic acid, L-alaninamide, L-alanine, L-alanyl glycine, L-arabinose, L-asparagine, L-fucose, L-histidine, L-leucine, L-phenylalanine, L-pyroglyutamic acid, malonic acid, *myo*-inositol, *N*-acetyl-D-galactosamine, *p*-hydroxyphenylacetic acid, propionic acid, quinic acid, sebacic acid and xylitol. Further phenotypic characteristics are given in the species description.

Flexirubin-type pigments were not detected. Crude cell extracts from the strains had identical adsorption spectra to that of *Cellulophaga lytica* with peaks at 264, 334, 451 and 478 nm and a shoulder at 425 nm. Two pigments with typical carotenoid-like spectra were separated by TLC of acetone extract. One orange pigment had adsorption peaks at 451, 477 and 503 nm, and one yellow pigment had adsorption peaks at 431 and 451 nm and a shoulder at 475 nm. The chromatography profile and adsorption patterns of pigments from the basaltic strains were identical to those of *C. lytica*. *C. lytica* pigments are identified as zeaxanthin (Aasen & Ljaen, 1966; Lewin & Lounsbey, 1969). The mean DNA G + C content of the four new strains was 31.4 ± 0.6 mol% (SD). The cellular fatty acid profile of strain H35^T is summarized in Table 1. The predominant fatty acids were 15:0 iso, 15:1 iso, 15:0 iso 3-OH and 17:0 iso 3-OH. The branched fatty acids accounted for 89 % of all identified fatty acids. The quinone analysis showed that 99 % of the quinones were menaquinone-6 and 1 % menaquinone-7.

The phylogenetic affiliation of the four new strains was examined by 16S rRNA gene sequence analysis (Fig. 3). Strain H35^T formed a clade together with *Gaetbulibacter saemankumensis* and *Algibacter lectus*. Levels of 16S rRNA gene sequence similarity to the type strains of these two species were almost equal, 94.3 and 94.8 %, respectively. H35^T also showed high 16S rRNA gene sequence similarity to the type strains of members of the genera *Formosa* (*Formosa algae*, 93 %), *Bizionia* (*Bizionia paragorgiae*, 94.4 %), *Lacinutrix* (*Lacinutrix copepodicola*, 93.9 %) and *Winogradskyella* (*Winogradskyella eximia*, 93.6 %), which were placed in a clade separate from that containing H35^T. The three algorithms used for construction of the phylogenetic trees generated the same branching in the *Algibacter*–*Gaetbulibacter*–*Formosa*–*Bizionia* clade to which strain H35^T is affiliated, but neighbour-joining bootstrapping values indicated that this tree had low bootstrapping values at some key branching points.

Fig. 2. Scanning electron micrograph of a cell of strain H35^T grown on FM at $22^{\circ}C$, showing a polar appendage structure (arrow). Bar, 0.2 μm .

Table 1. Fatty acid content of strain H35^T and the type species of some closely related reference genera

Taxa: 1, strain H35^T; 2, *Algibacter lectus* (data from Nedashkovskaya *et al.*, 2004); 3, *Gaetbulibacter saemankumensis* (data from Jung *et al.*, 2005); 4, *Formosa algae* (data from Ivanova *et al.*, 2004); 5, *Bizionia paragorgiae* (data from Nedashkovskaya *et al.*, 2005b); 6, *Lacinutrix copepodicola* (data from Bowman & Nichols, 2005); 7, *Winogradskyella eximia* (data from Nedashkovskaya *et al.*, 2005a). Fatty acids not found in strain H35^T and representing <1% of total fatty acid content in the other species are not represented.

Fatty acid	1	2	3	4	5	6	7
Straight chain							
14:0	0.2			1.03		0.4	
15:0		13.4	1.5	27.2		3.7	6.7
16:0	0.5		1.3	1.0		2.9	
Branched							
13:0 iso	0.2		2.0			0.1	
14:0 iso	0.8			0.3		1.4	1.4
15:0 iso	19.4	12.5	23.0	13.3	13.2	19.9	25.6
15:0 anteiso	6.2	7.2	10.8	7.2	12.3	18.2	7.0
15:1 iso	21.2	13.4	12.1	15.3	9.4	12.0	10.4
15:1 anteiso	1.7		1.5		5.8	14.2	1.4
16:0 iso	0.6			2.3	5.8	7.1	5.7
16:0 10 methyl							6.3
16:1 iso	0.5				5.4	4.6*	4.7
16:1 anteiso				1.0			
17:1 ω 9 <i>c</i> iso	0.6		2.2	1.4†			
17:1 ω 9 <i>c</i> anteiso			1.3	0.4‡			2.3§
Unsaturated							
15:1 ω 6 <i>c</i>	1.9	10.9		13.0		1.2	
15:1 ω 8 <i>c</i>				1.0			
16:1 ω 6 <i>c</i>				3.3			
16:1 ω 7 <i>c</i>						2.8	
17:1 ω 6 <i>c</i>	0.6		1.0				
17:1 ω 8 <i>c</i>	0.4			2.2			
18:1 ω 5 <i>c</i>	1.0						
Hydroxy							
14:0 3-OH				1.1			
15:0 2-OH	1.4		1.6				1.0
15:0 3-OH	2.8					0.2	
15:0 iso 3-OH	13.6	9.4	8.2			3.1	2.6
15:0 anteiso 3-OH						2.1	
16:0 3-OH	0.5						
16:0 iso 3-OH	4.5		2.0		6.8	2.7	3.2
17:0 2-OH	1.0		2.4				1.0
17:0 3-OH	0.4						
17:0 iso 3-OH	13.3	9.1	15.2			2.3	6.7
17:0 anteiso 3-OH						1.7	
Cyclic							
17:0 cyclo				7.4			2.4
Summed feature 3¶	4.9		10.4		6.3		6.1
Unknown 16:582#	1.2						
Unknown							5.6
% Total identified	99.4	75.9	86.1	98.43	58.7	100.6	88.4

*Branching unknown, iso and/or anteiso.

†Reported by Ivanova *et al.* (2004) as 17:1 iso.

‡Reported by Ivanova *et al.* (2004) as 17:1 anteiso.

§Reported by Nedashkovskaya *et al.* (2005a) as 17:1 anteiso.

||Position of unsaturation unknown.

¶Summed feature 3 represents the two fatty acids 15:0 iso 2-OH and/or 16:1 ω 7*c*, which could not be separated.

#Unknown 16:582 represents an unidentified fatty acid with an equivalent chain length of 16:582.

Fig. 3. Phylogenetic tree based on 16S rRNA gene sequences showing the position of basaltic strains H32, H33, H34 and H35^T and the type strains of closely related species. The tree was constructed based on a distance matrix and a neighbour-joining algorithm. An asterisk indicates that the branching was also resolved by the maximum-likelihood and maximum-parsimony algorithms. Numbers in parentheses are sequence accession numbers. Bootstrap values are given on the nodes of the tree. Bar, 0.01 substitutions per nucleotide position.

Menaquinone-6 was the major respiratory quinone in cells of strain H35^T, constituting 99 % of the total quinones. The basaltic strains also lacked the ability to hydrolyse cellulose. These two characteristics (Bernardet *et al.*, 2002), together with results from 16S rRNA gene sequence analysis, indicate that the strains belong within the family *Flavobacteriaceae*. Strain H35^T showed less than 97 % 16S rRNA gene sequence similarity to any recognized species and therefore it is likely to represent a novel species (Stackebrandt & Goebel, 1994). Based on the phylogenetic analysis (Fig. 3), H35^T might be placed within one of two genera, *Gaetbulibacter* or *Algibacter*. The fatty acid profile of H35^T shows many similarities with that of *Gaetbulibacter saemankumensis* (Table 1). However, several of the phenotypic characteristics of the basaltic strains differ from those of members of the genera *Gaetbulibacter* and *Algibacter* (Table 2), the most important difference being that the

basaltic strains are obligately aerobic, whereas *Gaetbulibacter* and *Algibacter* species are capable of fermentation/anaerobic respiration.

The basaltic strains also show high 16S rRNA gene sequence similarity to members of the genera *Bizionia*, *Lacinutrix* and *Winogradskyella*. Phylogenetic analysis, however, does not support the placement of the strains in these genera (Fig. 3), which is corroborated by several phenotypic and chemotaxonomic characteristics (Table 2) such as motility, and temperature and salinity requirements.

Based on these results, we suggest that the four strains should be included in the family *Flavobacteriaceae* (Bernardet *et al.*, 2002) as representatives of a novel species in a new genus, for which the name *Flaviramulus basaltis* gen. nov., sp. nov. is proposed.

Table 2. Differential characteristics of strain H35^T and the type species of other phylogenetically related genera

Taxa: 1, strain H35^T; 2, *Algibacter lectus* (data from Nedashkovskaya *et al.*, 2004); 3, *Gaetbulibacter saemankumensis* (data from Jung *et al.*, 2005); 4, *Formosa algae* (data from Ivanova *et al.*, 2004); 5, *Bizionia paragorgiae* (data from Nedashkovskaya *et al.*, 2005b); 6, *Lacinutrix copepodicola* (data from Bowman & Nichols, 2005); 7, *Winogradskyella eximia* (data from Nedashkovskaya *et al.*, 2005a). +, Positive; –, negative; ND, not determined.

Characteristic	1	2	3	4	5	6	7
Oxidase	–	+	+	–	+	ND	+
Motility	+	+	+	+	–	–	+
Requirement for growth factors	+	ND	ND	–	ND	–	ND
Temperature supporting growth (°C)							
0	+	–	–	–	–	+	–
30	+	+	+	+	+	–	+
40	–	–	+	+	–	–	–
Salinity supporting growth							
0.5 % NaCl	+	–	–	+	–	–	–
6 % NaCl	+	+	+	+	+	+	–
7 % NaCl	–	–	+	–	+	+	–
Metabolism*	A	F	F	F	A	A	A
Acid produced from carbohydrates	+	+	+	ND	–	–	+
Hydrolysis of:							
Agar	–	+	ND	–	–	–	ND
Aesculin	+	ND	+	ND	ND	–	ND
Gelatin	+	+	–	+	+	+	+
Starch	+	+	+	+	–	–	+
DNA	+	–	ND	ND	–	–	–
Urea	–	ND	–	+	–	–	–
Tween 20	+	+	+	ND	–	ND	+
Tween 40	+	+	–	+	+	ND	+
Tween 80	+	–	–	ND	+	+	–
H ₂ S production from cysteine	+	–	ND	ND	+	–	+
Mean DNA G+C content (mol%) (T _m)	31	32	34	34	38	37	36

*A, aerobic; F, fermentative.

Description of *Flaviramulus* gen. nov.

Flaviramulus (Fla.vi.ra'mu.lus. L. adj. *flavus* yellow; L. masc. nom. n. *ramulus* small branch, dim. of *ramus* branch or of things having a branching form; N.L. masc. n. *Flaviramulus* small yellow branch).

Cells are Gram-negative. Morphology varies with age of culture. Cells in exponential growth phase are rods, 0.2–0.3 µm in diameter and 1–3 µm in length. Cells in stationary phase are pleomorphic, 0.2–0.3 µm in diameter but with lengths ranging from 1 to 30 µm. Branched and curled cells are also seen in stationary-phase cultures. As cultures age, cells degenerate into spheroplasts ranging from 0.15 to 1 µm in diameter. Cells are motile, obligately aerobic heterotrophs. Cells contain yellow and orange carotenoids.

Flexirubin-type pigments are not found. Menaquinone-6 is the major respiratory quinone. Phylogenetically, this is a member of the family *Flavobacteriaceae*, class *Flavobacteria*, phylum *Bacteroidetes*. The type and only species is *Flaviramulus basaltis*.

Description of *Flaviramulus basaltis* sp. nov.

Flaviramulus basaltis (ba.sal'tis. L. masc. gen. n. *basaltis* of basalt, pertaining to the source of isolation).

Has the following properties in addition to those given for the genus. Colonies grown on MA are shiny, dark yellow, circular, convex, with an entire margin. Cells are catalase-positive, oxidase-negative and alkaline phosphatase-positive. Growth occurs from –2.0 to 34.0 °C, with optimum growth at 17.5–22.8 °C. The salinity range for growth is 3–60 g sea salts l^{–1}, with an optimum of 24–60 g l^{–1}. Growth is supported at pH 6.5–8.6, with optimum growth at pH 6.5–8.2. Requires seawater, yeast extract or thiamine and amino acids for growth. Utilizes and produces acids from several sugars. Utilizes organic acids and amino acids. Does not utilize alcohols. Hydrolyses L-tyrosine, aesculin, carrageenan, gelatin, starch, DNA, urea, and Tweens 20, 40 and 80, but not agar, cellulose or chitin. Produces H₂S from cysteine. Mean DNA G + C content is 31.4 ± 0.6 mol% (SD) as determined by the thermal denaturation method. Branched fatty acids are predominant.

The type strain, H35^T (= CIP 109091^T = DSM 18180^T), was isolated from seafloor basalt offshore of Jan Mayen in the Norwegian/Greenland Sea at a depth of 1300 m.

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